SNI

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Standar Nasional Indonesia

# Cocoa oilcake



ICS 67.140.30

Badan Standardisasi Nasional

# Contents

# Foreword

Standar Nasional Indonesia (SNI) *Cocoa oilcake,* is a new SNI.

The purpose of this standard-setting is:

- Protecting the health of consumers;
- Ensuring food trade run fairly and responsibly;
- Diversivication of products or product development;
- Support the development of cocoa processing industry and cocoa oilcake users industry.

This standard is formulated by considering the things listed in:

1. Undang-undang Republik Indonesia No. 5 Tahun 1984 tentang Perindustrian = Republic Indonesia Act No. 5 / 1984 on Industry.
2. Undang-undang Republik Indonesia No. 23 Tahun 1992 tentang Kesehatan = Republic Indonesia Act No. 23 of 1992 on Health.
3. Undang-undang Republik Indonesia No. 7 Tahun 1996 tentang Pangan = Republic Indonesia Act No. 7 / 1996 on Food.
4. Undang-undang Republik Indonesia No. 8 Tahun 1999 tentang Perlindungan Konsumen = Republic Indonesia Act No. 8 / 1999 on Consumer Protection.
5. Peraturan Pemerintah No. 69 Tahun 1999 tentang Label dan Iklan Pangan.= Government Regulation no. 69 of 1999 on Food Labels and Advertising.
6. Peraturan Pemerintah No. 28 Tahun 2004 tentang Keamanan, Mutu dan Gizi Pangan = Government Regulation no. 28 of 2004 on Security, Food Quality and Nutrition.
7. Keputusan Direktorat Jenderal Pengawasan Obat dan Makanan No.03725/B/SK/VII/89 tentang Batas Maksimum Cemaran Logam Berat dalam Makanan dan Minuman atau revisinya = Decision of Directorate General of Drug and Food Control No.03725/B/SK/VII/89 on Maximum Limit of Heavy Metal Contamination in Food and Beverage or its revisions.
8. Keputusan Direktorat Jenderal Pengawasan Obat dan Makanan No. 03726/B/SK/VII/89 tentang Batas Maksimum Cemaran Mikroba dalam Makanan dan Minuman atau revisinya = Decision of Directorate General of Drug and Food No.03726/B/SK/VII/89 on Maximum Limit of Microbial Contamination in Food and Beverage or its revisions.

This standard is formulated by the Technical Committee 67 - 04 Foods and Beverages. This standard had been discussed through the technical meetings and agreed in Pre-consensus meeting, and Consensus meeting on December 19, 2008 in Jakarta. Attending in the meeting were representatives from consumers, manufacturers, testing agencies, sience and technology agencies, and other related agencies.

This standard has been passed through the process of e-balloting on June 22, 2009 until August 22, 2009 with results of RASNI.

# Cocoa cake

## 1 Scope

This standard set the terms and definitions, composition, quality requirements, sampling, and test methods for cocoa oilcake.

## 2 Normative references

SNI 19-0428-1998, *Petunjuk pengambilan contoh padatan* (Solid Sampling Instructions)

## 3 Terms and definitions

**3.1**
***Cocoa oilcake***
cocoa products obtained from the separation of part or all of the fat from cocoa nibs or cocoa mass

**3.2**
**Cocoa nibs (cocoa beans pieces)**
cocoa beans which its skin have been removed

**3.3**
**cocoa beans**
cocoa beans *(Theobroma cacao* L.) which has been cleaned and dried

**3.4**
**cocoa mass**
pasta products obtained from cocoa nibs (cocoa bean pieces) by milling without removing its fat

## 4 Composition

### 4.1 The main raw materials

Cocoa nibs or cocoa mass

### 4.2 Food additives

Food additives permitted to cocoa oilcake accordance with applied regulations.

## 5 Quality requirements

**Tabel 1 — Quality requirements of cocoa oilcake**

| No | Testing Criteria | Units | Terms |
|---|---|---|---|
| 1 | State | | |
| 1.1 | Smell | - | normal (typical cocoa oilcake) |
| 1.2 | Taste | - | normal (typical cocoo oilcake) |
| 1.3 | Color | | Brown to black |
| 2 | Content of the skin (based on fat-free dry material) (w / w) | % | max. 1.75 |
| 3 | Water content (w / w) | % | Max. 5,0 |
| 4 | Metal contamination | | |
| 4.1 | Lead (Pb) | mg / kg | Max. 1,0 1.0 |
| 4.2 | Cadmium (Cd) | mg / kg | max. 0,5 0.5 |
| 4.3 | Mercury (Hg) | mg / kg | max. 0.03 |
| 4.4 | Tin (Sn) | mg / kg | max. 40.0 |
| 5 | Arsenic contamination (As) | mg / kg | max. 1.0 |
| 6 | Microbial contamination | | |
| 6.1 | Total plate numbers | colonies / g | max. $1 \times 10^4$ |
| 6.2 | *Escherichia coli* | APM / g | <3 |
| 6.3 | *Salmonella sp.* | -- | negative / 25 g |
| 6.4 | Molds and yeasts | colonies / g | max. $1 \times 10^2$ |

## 6 Sampling

Sampling methods is accordance with SNI 19-0428-1998.

## 7 Test methods

Test methods for cocoa oilcake are like below:

a. Sample preparation in accordance with Appendix A.1.
b. Test methods in accordance with A.2.
   - Smell test methods in accordance with Appendix A.2.1.
   - Taste test methods in accordance with Appendix A.2.2.
   - Color test methods in accordance with Appendix A.2.3.
c. Test methods for the content of the skin (based on fat-free dry material) in accordance with Appendix A.3.
d. Test methods for water content level in accordance with Appendix A.4.
e. Test methods for metal contamination in accordance with Appendix A.5.
   - Test methods for cadmium (Cd) and lead (Pb) in accordance with Appendix A.5.1.
   - Test methods for mercury (Hg) in accordance with Appendix A.5.2.
   - Test methods for tin (Sn) in accordance with Appendix A.5.3.
f. Test methods for arsenic contamination (As) in accordance with Appendix A.6.
g Test methods for microbial contamination in accordance with Appendix A.7.

- Preparation and homogenization of samples in accordance with Appendix A.7.1.
- Test methods for total plate numbers in accordance with Appendix A.7.2.
- Test methods for *Escherichia coli* test method in accordance with Appendix A.7.3.
- Test methods for *Salmonella sp.* in accordance with Appendix A.7.4.
- Test methods for molds and yeasts in accordance with Appendix A.7.5.

## 8 Tested Conditions

Products considered to have passed the test if it meets the quality requirements according to Article 4.

## 9 Hygiene

The methods to produce hygienic products, including its preparation and handling are in accordance with the applied terms on the Guidelines in Production of Good Processed food (Pedoman Cara Produksi Pangan Olahan yang Baik)

## 10 Packaging

Cocoa oilcake is packed in a sealed container which is not affected or affect the content, and safe for storage and transport.

## 11 Labeling requirements

Labeling requirements in accordance with the applied regulation on food labels and advertisements.

# Appendix A
(normative)

## Cocoa oilcake test methods

### A.1 Sample Preparation

Sample preparation consists of preparing samples for microbiological test, organoleptic test and chemical analysis. Sampling for microbiological test carried out first, then followed by taking samples for organoleptic test and chemical analysis.
Open cocoa oilcake package aseptically then stir and take 400 g cocoa bungkil samples for microbiological test. Move into a sample bottle, which is clean, sterile, and tightly closed. Stir again and take the cocoa oilcake sufficiently for organoleptic test  then move into a sample bottle which is clean and tightly closed. Stir again and take 500g of cocoa oilcake for chemical analysis and then move into a bottle sample which is clean and tightly closed

### A.2 State

#### A.2.1 Smell

##### A.2.1.1 Principles

Conduct an analysis of the organoleptic test sample using sense of smell.

##### A.2.1.2 Workings

a. Take the test samples that have been prepared sufficiently and place it on a clean watch glass and dry,
b. Smell the test samples to find out the smell, and
c. Do work at least by 3 panelist or 1 expert.

##### A.2.1.3 Methods of stating results

a. If the typical smell of cocoa oilcake is smelt, the results are stated "normal"; and
b. if other small than the typical smell of cocoa oilcake is smelt, then the results are stated "not normal".

#### A.2.2 Taste

##### A.2.2.1 Principle

Conduct an analysis of the organoleptic test sample using taste senses.

##### A.2.2.2 Workings

a) Take the test samples that have been prepared sufficiently and feel with  tongue, and
b) do work at least by 3 panelist or 1 expert.

### A.2.2.3 Methods of stating results

a. If the typical taste of cocoa oilcake is felt, then the results are stated "normal"; and

b. if other taste than the typical cocoa oilcake is felt, then the results are stated "not normal".

### A.2.3 Color

### A.2.3.1 Principle

Conducted an analysis of the organoleptic test samples by using the senses of vision.

### A.2.3.2 Workings

a) Take the test samples that have been prepared sufficiently and place it on a clean and dry watch glass,

b) observe the color of test samples, and

c) do work at least by 3 panelist or 1 expert.

### A.2.3.3 Methods to state results

a) If brown color is seen, then the results are stated "normal"; and

b) if color other than brown is seen, mention the color observed and the result are stated "not normal".

## A.3 Content of the skin (based on fat-free dry material)

### A.3.1 Principle

Destruct the organic compounds contained in test samples without destructing stone cells using the reagents Bellucci that is boiled for 10 minutes. The stone cells separated by centrifugation an then suspended in glycerol. The number of stone cells groups and number of stone cells in each group are observed and counted using microscope. Content of the skin is counted by determining the empirical calibration curve that gives the relationship between the number of stone cell groups and the average size of the test sample group with content of skin 1% which is measured in fat-free dry ingredients.

### A.3.2 Tools

a) Microscope;
b) *counting grid,* size ± (33 x 33 x 0.2) mm;
c) calibrated analytical scale/balance with accuracy 0.1 mg;
d) centrifuges;
e) calibrated oven with accuracy 1 ° C;
f) water bath;
g) culture tube;
h) glass tube pointy toes, base diameter of ± 5 mm, top diameter of 1 mm, and length 150 mm;
i) glass object, size ± (75 x 38 x 0.5) mm; and
j) Beaker glass.

### A.3.3 Reagents

a) Reagents Bellucci;
(glacial acetic acid: concentrated $HNO_3$ : distilled water = 36: 5: 9)
b) glycerol; and
c) ether.

### A.3.4 Workings

#### A.3.4.1 Remove fat

a) Gerus cocoa oilcake using mortar until smooth and strain using a 30 mesh sieve,
b) weigh 15 g filtered cocoa oilcake into a 250 ml centrifuge tubes,
c) add 100 ml of ether for fat extraction,
d) close the tube and shake until the fat soluble in ether and then filter,
e) wash the filter with ether,
f) Centrifugate the solution for 10 minutes at 2000 rpm and discard the supernatan
g) add 100 ml of ether for extraction and re-done in accordance with A.3.4.1.d,
h) add 100 ml of ether, close the tube, and shake,
i) immediately pour into *a rotary evaporator* flask and do evaporation of ether for about 20 minutes until dry,
j) remove all residues into the mortar using a spatula or clean and dry spoon then gerus until smooth,
k) after a smooth, move back into aluminum or porcelain dish and allow to dry for 10 minutes to 15 minutes in a water bath to remove the remaining ether, and
l) 00 °C selama 1 jam sehingga diperoleh bungkil kakao kering bebas lemak. dry in oven at a temperature of 100 ° C for 1 hour so that fat-free dry cocoa oilcake obtained.

#### A.3.4.2 Calculate the content of the skin

a) Weigh accurately 0.500 g (W) fat-free dry cocoa oilcake into a 150 ml Beaker glass
b) add 20 ml of reagents Bellucci slowly and while stirring,
c) Rinse wall of the Beaker glass with the remaining reagents Bellucci if there are cocoa oilcake patched,
d) boil for 10 minutes and stir occasionally then chill for ± 5 minutes,
e) weigh culture tubes inside 30 ml Beaker glass that serves as a buffer,
f) remove residue carefully into the culture tube with a little addition of water and centrifugation at full speed for about 3 minutes,
g) throw away the supernatan liquid, add distilled water until ¾ the volume of the tube, cap tube and shake until the residue is dispersed in solution and centrifuged again at full speed for about 3 minutes,
h) throw away the supernatan again and add a solution of glycerol (glycerol: water = 3: 1) until the tube and buffer contents weighed (20 ± 0.03) g (L),
i) shake vigorously until completely mixed and transferred to a vial containing a small magnetic bar, leave the bottle for 5 minutes to 10 minutes until the bubbles disappear,
j) weigh simultaneously the scale *slide* glass and it lid,
k) stir the solution in the bottle using a magnetic stirrer for 1 minute at maximum speed until no bubbles formed,

l) remove drops of solution with a spoon (0.04 ± 0.01) g (D) into the middle of the *slide glass,* place the lid above *the slide* so that the solution will move to the edge of *the slide,* not to press the lid,

m) weigh *slide* up to 0.1 mg,

n) cap bottle with a rubber cover to prevent evaporation,

o) place *slide* on a microscope *slide* and count *stone cell* by *scanning* 100 times and count *stone cell* in ≥ 200 times,

p) count alll *stone cell* either alone or in groups, or that is damaged with cell size ≥ 0.5 (C), do not count the small fragments,

q) make observations with the microscope as much as two times for each job, and

r) do the duplo job.

### A.3.5 Calculation

$$\text{Bobot bungkil kakao dalam tetesan larutan (M) (mg)} = 1000\ \frac{WD}{L}$$

$$\text{Kandungan kulit (S) (\%)} = \frac{84\,C}{17200M - C}$$

**Description:**

W is the weight of fat-free dry cocoa oilcake, expressed in grams (g);

L is the weight of solution fat-free dry cocoa oilcake solution, expressed in grams (g);

D is the weight of the solution droplets are counted, expressed in grams (g);

M is the weight of fat free dry cocoa oilcake in droplets solution, expressed in milligrams (mg);

C is the number of *stone cells* in liquid droplets and

S is the content of the skin, expressed in percent (%);

### A.3.6 Accuracy

The range results of twice test is a maximum of 10% of the average value of the content of the skin. If the range is greater than 10%, then the analysis must be repeated again.

## A.4 Water content

### A.4.1 Principle

Water content is calculated based on the weight lost during heating in an oven at a temperature (100 ± 2) ° C.

### A.4.2 Tools

a) Oven calibrated with accuracy 1 ° C;

b) calibrated analytical balance with accuracy 0.1 mg;

c) desiccator containing desiccant; and

d) nickel dish, platinum or aluminum lids.

### A.4.3 Workings

a) Heat the dish and its lid in the oven at a temperature (100 ± 2) ° C for about an hour and chill in desiccatorr for 20 minutes to 30 minutes, then weigh using analytic balance (dish and its lids) ($W_0$)

b) enter a 2 g sample into a dish, cover, and weigh ($W_{1)}$,

c) reheat the dish containing the sample was open with the dish lid placed beside the dish in the oven at a temperature (100 ± 2) ° C for 3 (three) hours after the oven temperature (100 ± 2) ° C,
d) cover the dish while still in the oven, move immediately into the desiccator and chill for 20 to 30 minutes and then weigh it,
e) lakukan pemanasan kembali selama 1 jam dan ulangi kembali sampai perubahan bobot antara pemanasan selama 1 jam mempunyai interval ≤ 2 mg ($W_2$ ), do reheating for 1 hour and repeat again until the change of weight between heating for 1 hour has interval ≤ 2 mg ($W_{2)}$,
f) do the duplo job, and
g) calculate the water content in the sample.

### A.4.4 Calculation

$$\text{Kadar air (\%)} = \frac{W_1 - W_2}{W_1 - W_0} \times 100\%$$

**Description:**

$W_0$ is the weight of empty dish and lid, expressed in grams (g);

$W_1$ is the weight of the dish, lid and sample before drying, expressed in grams (g);

$W_2$ is the weight of the dish, lid and sample after drying, expressed in grams (g).

### A.4.5 Accuracy

The result range of twice test a maximum of 5% of the average value of moisture content. then the analysis must be started again.

## A.5 Metal contamination

### A.5.1 Determination of metal contamination of cadmium (Cd) and lead (Pb)

#### A.5.1.1 Principle

Destruction of samples by dry ashing at 450 ° C, followed by dissolving in acid. Dissolved metal is calculated using Atomic Absorption spectrophotometer (AAS) with a maximum wave length of 228.8 nm for Cd and 283.3 nm for Pb.

#### A.5.1.2 Tools

a) Atomic absorption spectrophotometer (AAS) with its calibrated tools (Cd cathode lamps and Pb) calibrated (preferably using graphite furnace AAS);
b) calibrated furnace with accuracy 1 ° C;
c) calibrated analytical balance with accuracy 0.1 mg;
d) electric bath;
e) water bath;
f) 0.05 ml scale measuring pipette or calibrated micro burette;
g) calibrated volumetric flask 50 ml, 100 ml, 1000 ml
h) measuring cup capacity 10 ml; and
i) Beaker glass 250 ml.
j) porcelain / platinum / quartz cup with a capacity of 50 ml to 100 ml;
k) *polyprophylene* container;
l) kertas saring tidak berabu dengan spesifikasi *particle retention liquid* 20 – 25 µm; filter paper without ash with specification of *particle retention liquid* from 20 to 25 µm;

### A.5.1.3 Reagents

a) Solution of nitric acid, concentrated $HNO_3$ (65%, Bj 1.4);
b) hydrochloric acid solution, concentrated HCl (37%, Bj 1.19);
c) solution of nitric acid, $HNO_3$ 0.1 N;
dilute 7 ml of $HNO_3$ 65% with distilled water in a 1000 ml volumetric flask and dilute to the mark line.
d) solution of hydrochloric acid, HCl 6N;
e) dilute 500 ml HCl 37% with distilled water in a 1000 ml volumetric flask and dilute to the mark line.
f) Standard solution of 1000 µg / ml Cd;
g) dissolve 1.000 g of Cd with 7 ml of concentrated $HNO_3$ in 250 ml Beaker glass and put into 1000 ml volumetric flask then dilute with distilled water until the mark lines. Alternatively, can use standard solution Cd 1000 µg / ml that ready to use.
h) standard solution of 200 µg / ml Cd;
i) pipette 10 ml of standard solution of 1000 µg / ml Cd into 50 ml volumetric flask and then dilute with distilled water to the mark line and then whipp. This second standard solution has a concentration of 200 µg / ml Cd.
j) standard solution of 20 µg / ml Cd;
k) pipette 10 ml standard solution of 200 µg / ml of Cd into the 100 ml volumetric flask and then dilute with distilled water to the mark line and then whipp. This third standard solution has a concentration of 20 µg / ml Cd.
l) working standard solution of Cd;
m) pipette into a 100 ml volumetric flask each of 0 ml, 0.5 ml, 1 ml 2; ml; 4 ml 7 ml and 9 ml of standard solution of 20 µg / ml and then add 5 ml $HNO_3$ solution 1 N or HCl 6 N, and dilute with distilled water to the mark line and then whipp. This working standard solution has a concentration of 0 µg / ml; 0.1 µg / ml; 0.2 µg / ml; 0.4 µg / ml; 0.8 µg / ml; 1.4 µg / ml and 1.8 µg / ml Cd.
n) standard solution of 1000 µg / ml Pb;
o) dissolve 1.000 g of Pb with 7 ml of concentrated $HNO_3$ in 250 ml Beaker glass and put into 1000 ml volumetric flask and dilute with distilled water until the line mark. Alternatively, can be used ready to use standard solution Pb 1000 µg / ml.
p) standard solution of 50 µg / ml Pb; and
q) Pipette 5.0 ml of standard solution of 1000 µg / ml Pb in 100 ml volumetric flask and dilute with distilled water until the line mark and then whipped. This second standard solution has a Pb concentration of 50 µg / ml.
r) Pb working standard solution;
s) Pipette into a 100 ml volumetric flask each of 0 ml, 0.2 ml; 0.5 ml; 1 ml 2 ml; 3 ml and 4 ml of standard solution 50 µg / ml and add 5 ml solution of $HNO_3$ 1 N or HCl 6 N, and dilute with distilled water until the line mark and then whipped. This working standard solution has a concentration of 0 µg / ml; 0.1 µg / ml; 0.25 µg / ml; 0.5 µg / ml; 1.0 µg / ml; 1.5 µg / ml and 2.0 µg / ml Pb.

### A.5.1.4 Workings

a) Weigh 10 g to 20 g of sample (m) accurately in a porcelain / platinum / quartz cup;
b) place the cup containing the sample test on electric bath and heat gradually until the test sample did not smoke anymore;
c) ashing in the furnace (450 ± 5) ° C until white ash, free from carbon;
d) if ash not yet free from carbon marked with grayish color, wet with a few drops of water and add drop by drop of concentrated $HNO_3$ approximately 1 ml to 3 ml;

e) drain the cup on electrical bath and put back into the furnace at a temperature of 450 ° C and continue heating until a whitw ash . The addition of concentrated $HNO_3$ can be repeated if the ashes are still grayish;

f) dissolve white ash 5 ml 6 N HCl, while heated in an electric bath or water bath until dry, then dissolved with $HNO_3$ 0.1 N and enter into a 50 ml volumetric flask until markline with distilled water (V ). If necessary, strain the solution using free of ash filter paper with specification of *liquid particle retention from 20 to* 25 µm into the container *polyprophylene;*

g) prepare blank solution with the addition of reagents and the same treatment as an example;

h) read absorbans working standard solution and sample solution to blank using the AAS at the maximum wave length of about 228.8 nm for Cd and 283.3 nm for Pb,

i) create a calibration curve between the metal concentrations (µg / ml) as the Y axis,

j) plot the reding results of sample solution to the calibration curve (C), and

k) calculate metals content in the sample.

### A.5.1.5 Calculation

$$\text{Kandungan logam (mg/kg)} = \frac{C}{m} \times V$$

**Description:**

C is the metal concentration from the calibration curve, expressed in micrograms per milliliter (µg / ml);

V is the volume of final solution, expressed in milliliters (ml);

m is the weight of sample, expressed in grams (g).

### A.5.1.6 Accuracy

The range of twice test results is a maximum 16% of the average value of metal content result. If the range is greater than 16%, then the analysis must be started again.

## A.5.2 Determination of mercury (Hg)

### A.5.2.1 Principle

The reaction between mercury compounds with $NaBH_4$ or $SnCl_2$ in acidic conditions to form the Hg atomic gases. The number of Hg formed is proportional to the absorbans Hg read using Atomic Absorption Spectrophotometer without a flame at the maximum wavelength of 253.7 nm.

### A.5.2.2 Tools

a) atomic absorption spectrophotometer (AAS) which is equipped with Hg cathode lamps and hydride vapor generator ( "HVG");

b) calibrated analytical balance with accuracy 0.1 mg;

c) electric bath;

d) destruction flask 250 ml with rounded base;

e) calibrated volumetric flask 100 ml, 500 ml and 1000 ml

f) measuring pipette scale 0.05 ml or calibrated micro burette;

g) cooler made of borosilicate, diameter 12 mm to 18 mm, 400 mm high filled with Raschig rings as high as 100 mm, and covered with boiling stones 4 mm in diameter above 20 mm high ring;

h) 25 ml measuring cup.

### A.5.2.3 Reagents

a) Sulfuric acid, $H_2SO_4$ 9 M;
b) nitric acid, $HNO_3$ 7 M;
c) Boiling stones;
d) mixture of $HNO_3$: $HClO_4$ (1:1);
e) hydrogen peroxide, $H_2O_2$;
f) sodium molibdat solution 2%.
g) reducing solution;
h) mix 50 ml of $H_2SO_4$ with 300 ml of distilled water in 500 ml Beaker glass and let cool until room temperature and add 15 g NaCl, 15 g hidroksilamin sulfate, and 25 g $SnCl_2$. Move into 500 ml volumetric flask and dilute with distilled water until the line mark.
i) solution of $NaBH_4$;
j) dissolve 3 g of powdered $NaBH_4$ and 3 g NaOH with distilled water in a 500 ml volumetric flask.
k) diluent solution;
l) enter up 300 ml to 500 ml distilled water into 1000 ml volumetric flask and add 58 ml of $HNO_3$ and 67 ml of $H_2SO_4$. Dilute with distilled water until the line mark and whipped.
m) standard solution of 1000 µg / ml Hg;
n) dissolve 0.1354 g of $HgCl_2$ with approximately 25 ml of distilled water in 250 ml Beaker glass and put into 100 ml volumetric flask and dilute with distilled water until the line mark.
o) standard solution of 1 µg / ml of Hg; and
p) pipette 1 ml standard solution 1000 mg / l Hg in 1000 ml volumetric flask and dilute with a diluent solution to the line mark and then whipped. This second standard solution has a concentration of 1 mg / l.
q) working standard solution of Hg;
r) pipette each 0.25 ml 0.5 ml 1 ml and 2 ml of standard solution 1 mg / l to 100 ml separate volumetric flask and dilute with a diluent solution to the line mark . This working standard solution has a concentration of 0, 0025 µg / ml; 0.005 µg g / ml; 0.01 mcg / ml; 0.02 µg / ml Hg.

### A.5.2.4 Workings

#### A.5.2.4.1 wet ashing

a) Weigh 5 g of sample (m) carefully into the destruction flask and add 25 ml of $H_2SO_4$ 9 M, 20 ml $HNO_3$ 7 M, 1 ml solution of sodium molibdat 2%, and 5 to 6 boiling stone
b) Connect the destruction flask with the cooler and heat on electric bath for 1 hour. Stop heating and leave for 15 minutes,
c) add 20 ml mixture of $HNO_3$ - $HClO_4$ (1:1) through the cooler,
d) stop the flow of water in the cooling and heat over high heat until white fumes appear. Continue heating for 10 minutes and chill,
e) add 10 ml of distilled water through the cooler carefully shaken with pumpkin-toss,
f) boil again for 10 minutes,
g) turn off the heating and wash cooler with 15 ml of distilled water for 3 times and then let cool until room temperature,
h) move the solution destruction sample into 100 ml volumetric flask quantitatively and dilute with distilled water until the line mark (V),
i) pipette 25 ml of solution above into the 100 ml volumetric flask and dilute degan diluent solution until the line mark,

j) Prepare blank solution with reagent addition and the same treatment as sample,
k) add reducing solution into the Hg working standard solution, sample solution and blank solution at the instrument "HVG",
l) read absorbans standard solution work, sample solution, and blank solution using the AAS without flame at 253.7 nm wavelength,
m) create a calibration curve between the metal concentrations (µg / ml) as the X axis and absorbans as Y axis
n) plot results of sample solution reading to the calibration curve (C),
o) do the duplo work, and
p) calculate Hg content in the sample.

#### A.5.2.4.2 Destruction using *microwave* or destruction of a closed system

a) Weigh 1 g of sample (m) into the tube destruction and add 5 ml $HNO_{3,}$ 1 ml $H_2O_2$ and then seal tightly
b) enter into a *microwave* oven and work in accordance with the devices use instruction
c) move the destruction solution sample into 50 ml volumetric flask quantitatively and dilute with distilled water until the line mark (V),
d) prepare blank solution with reagent addition and the same treatment as sample,
e) add reducing solution into the working standard solution, sample solution and blank solution at the instrument "HVG",
f) read absorbans working standard solution, sample solution, and blank solution using the AAS without flame at 253.7 nm wavelength,
g) create a calibration curve between the metal concentrations (µg / ml) as the X axis and absorbans as Y axis,
h) plot hasil pembacaan larutan contoh terhadap kurva kalibrasi (C), plot results of sample solution reading to the calibration curve (C),
i) do the duplo work, and
j) calculate Hg content in the sample.

### A.5.2.5 Calculation

$$\text{Kandungan merkuri (Hg) (mg/kg)} = \frac{C}{m} \times V \times fp$$

**Description:**

C is the concentration of metal from the calibration curve, expressed in micrograms per milliliter (µg / ml)
V is the volume of final solution, expressed in milliliters (ml);
m is the weight of sample, expressed in grams (g); and fp is the dilution factor.

### A.5.2.6 Accuracy

The range of twice test results a maximum 16% of the average value of mercury content (Hg) result. If the range is greater than 16%, then the analysis must be started again.

### A.5.3 Determination of tin (Sn)

#### A.5.3.1 Principle

sample is destructed with $HNO_3$ and HCl and then add KCl to reduce interference. Sn is read using atomic absorption spectrophotometer at the maximum wavelength of 235.5 nm by flame oxidation of $N_2H_2OC_2$.

#### A.5.3.2 Tools

a) Atomic absorption spectrophotometer (AAS) with its appliance (Sn cathode lamps) calibrated;
b) calibrated furnace with accuracy 1 ° C;
c) calibrated analytical balance with accuracy 0.1 mg;
d) electric bath;
e) water bath;
f) calibrated measuring pipette scale 0.1 ml capacity 5 ml and 10 ml calibrated;
g) erlenmeyer 250 ml;
h) calibrated volumetric flask 50 ml, 100 ml, and 1000 ml,
i) measuring cup capacity 50 ml; and
j) Beaker glass. 250 ml

#### A.5.3.3 Reagents

a) Potassium chloride, 10 mg / ml K;
dissolved 1.91 g KCl in water to be 100 ml.
b) concentrated nitric acid, concentrated $HNO_3$;
c) concentrated hydrochloric acid, concentrated HCl;
d) standard solution of 1000 mg / l Sn; and
dissolved 1000 g Sn in 200 ml of concentrated hydrochloric acid in 1000 ml volumetric flask, add 200 ml of distilled water, let cool at room temperature and dilute with distilled water until line mark
e) working standard solution of Sn.
pipette 10 ml of concentrated HCl and 1.0 ml of KCl solution into each 100 ml volumetric flask. Tambahkan masing-masing 0 ml; 0,5 ml; 1,0 ml; 1,5 ml; 2,0 ml dan 2,5 ml larutan baku 1000 mg/L Sn dan encerkan dengan air suling sampai tanda garis. Larutan baku kerja ini memiliki konsentrasi 0 µg/ml; 5 µg/ml; 10 µg/ml; 15 µg/ml; 20 µg/ml dan 25 µg/ml Sn. Add respectively 0 ml; 0.5 ml; 1.0 ml; 1.5 ml 2.0 ml and 2.5 ml of standard solution of 1000 mg / L Sn and dilute with distilled water until the mark lines. This working standard solutions has a concentration of 0 µg / ml; 5 µg / ml; 10 µg / ml; 15 µg / ml; 20 µg / ml and 25 µg / ml Sn.

#### A.5.3.4 Workings

a) Weigh 10 g to 20 g (m) carefully into Erlenmeyer 250 ml, add 30 ml concentrated $HNO_3$ and allow 15 minutes,
b) heat gently for 15 minutes in the closet acid, avoid the occurrence of excessive spark,
c) continue heating so that the remaining volume of 3 ml to 6 ml or until the sample began to dry on the bottom, avoid the formation of charcoal,
d) lift erlenmeyer from electric bath, add 25 ml of concentrated HCl, and heat up for 15 minutes until the explosion of steam $Cl_2$ stops,
e) increase heating and simmer so that the remaining volume is 10 ml to 15 ml,
f) add 40 ml of distilled water (V), stir, and pour into 100 ml volumetric flask, rinse the erlenmeyer with 10 ml of distilled water,
g) add 1.0 ml of KCl, let cool at room temperature, chop with distilled water and filtering,
h) prepare blank solution with addition of reagents and the same treatment as sample,

i) read absorbans of working standard solution and sample solution to blank using AAS at the maximum wavelength of 235.5 nm with flame oxidation of $N_2OC_2H_2$
j) create a calibration curve between the metal concentrations (µg / ml) as the X axis and absorbans as Y axis
k) plot results of sample solution reading to the calibration curve (C),
l) do the duplo work, and
m) compute Sn content in the sample.

### A.5.3.5 Calculation

$$\text{Kandungan timah (Sn) (mg/kg)} = \frac{C}{m} \times V$$

**Description:**

C is the concentration of lead from the calibration curve, expressed in micrograms per milliliter (µg / ml)
V is the volume of final solution, expressed in milliliters (ml);
m is the weight of sample, expressed in grams (g).

### A.5.3.6 Accuracy

The range of twice test results a maximum 16% of the average value of the content of tin (Sn). If the range is greater than 16%, then the analysis must be started again.

## A.6 Arsenic Contamination (As)

### A.6.1 Principle

Sample is destructed with acid to be arsenic solution. The solution $As^{5+}$ is reduced with KI to be $As^{3+}$ and reacted with $NaBH_4$ or $SnCl_2$ to form $AsH_3$ that then read with AAS at the maximum wavelength of 193.7 nm.

### A.6.2 Tools

a) Atomic absorption spectrophotometer (AAS) which is equipped with cathode lamp As and hydride vapor generator ( "HVG");
b) calibrated furnace with accuracy 1 ° C;
c) calibrated analytical balance with accuracy 0.1 mg;
d) electric heaters;
e) borosilicate flask round by 50 ml;
f) Kjeldahl flask 250 ml
g) calibrated volumetric flask 50 ml, 100 ml, 500 ml, 1000 ml and
h) volumetric pipette 25 ml;
i) porcelain cup capacity 50 ml;
j) measuring cup; and
k) Calibrated measuring Pipette scale 0.05 ml or micro burette.

### A.6.3 Reagents

a) Nitric acid, concentrated $HNO_3$;
b) perchloric acid, concentrated $HClO_4$;
c) natrium boronhidrida, $NaBH_4$ ; sodium boronhidrida, $NaBH_4$;
dissolve 3 g $NaBH_4$ and 3 g NaOH with distilled water until the line mark in volumetric flask 500 ml.

d) hydrochloric acid, HCl 8 M;
larutkan 66 ml HCl 37 % kedalam labu ukur 100 ml dan encerkan dengan air suling sampai tanda garis. dissolve 66 ml of HCl 37% into volumetric flask 100 ml and dilute with distilled water until the line mark .

e) tin (II) chloride, $SnCl_2.2H_2O$ 10%;
weigh 50 g $SnCl_2.2H_2O$ into the Beaker glass 200 ml and add 100 ml HCl 37%. Heat until the solution is clear and chill then pour into a 500 ml volumetric flask and dilute with distilled water until the line mark.

f) potassium iodide, KI 20%;
weigh 20 g KI in volumetric flask 100 ml and dilute with distilled water until the line mark (solution should be made immediately before use).

g) Solution of $Mg(NO_3)_2$ 75 mg / ml;
Dissolve 3.75 g of MgO with 30 ml of $H_2O$ carefully, add 10 ml $HNO_3$, chill and dilute to 50 ml with distilled water;

h) standard solution of 1000 µg / ml As;
larutkan 1,3203 g $As_2O_3$ kering dengan sedikit NaOH 20 % dan netralkan dengan HCl atau $HNO_3$ 1:1 (1 bagian asam : 1 bagian air). Masukkan ke dalam labu ukur 1 L dan encerkan dengan air suling sampai tanda garis. dissolve 1.3203 g $As_2O_3$ dry with a little of NaOH 20% and neutralize with HCl or $HNO_3$ 1:1 (1 part acid: 1 part water). Put it in a volumetric flask 1 L and dilute with distilled water until the line mark.

i) standard solution of 100 µg / ml As;
pipette 10 ml of arsenic standard solution 1 000 µg / ml to volumetric flask 100 ml and dilute with distilled water until the line mark . This second standard solution has a concentration of 100 µg / ml As.

j) standard solution of 1 µg / ml As; and
pipette 1 ml of arsenic standard solution 100 mg / l to 100 ml volumetric flask and dilute with distilled water until the line mark. This third standard solution has a concentration of 1 µg / ml As.

k) working standard solution As;
pipette each of 1.0 ml; 2.0 ml; 3.0 ml; 4.0 ml and 5.0 ml standard solution 1 µg / ml As to the volumetric flask 100 ml, and dilute separately with distilled water until the line mark then shake. This working standard solution has a concentration of 0.01 µg / ml; 0.02 µg / ml; 0.03 µg / ml; 0.04 µg / ml and 0.05 µg / ml As.

### A.6.4 Workings

#### A.6.4.1 Wet ashing

a) weigh 5 g to 10 g of sample (m) into a Kjeldahl flask 250 ml, add 5 ml to 10 ml of concentrated $HNO_3$ and 4 ml to 8 ml concentrated $H_2SO_4$ carefully;
b) heat and add concentrated $HNO_3$ little by little so that sample or colored brown or blackish,
c) add 2 ml of $HClO_4$ 70% little by little and heat again until the solution became clear or yellowish (if carchoaling occurred after the addition of perchloric acid, add a little more concentrated $HNO_3$),
d) let cool, add 15 ml of $H_2O$ and 5 ml of saturated oxalic ammonim,
e) heat until appear steam $SO_3$ in the flask neck,
f) let cool, quantitatively transferred into volumetric flask 50 ml and dilute with distilled water until the line mark (V),
g) pipette 25 ml of solution above and add 2 ml HCl 8 M, 0.1 ml KI 20% and then shake and allow at least 2 minutes,
h) prepare blank solution with the addition of reagents and the same treatment as sample,
i) add reducing solution ($NaBH_4$)into the working standard solution As, sample solution, and blank solution at the instrument "HVG",
j) read absorbans of working standard solution, sample solution, and blank solution using the AAS without flame at 193.7 nm wavelength,

k) create a calibration curve between the metal concentrations (µg / ml) as the X axis and absorbans as Y axis,
l) plot results of samples solution reading to the calibration curve (C),
m) do the duplo work, and
n) Count Acount the As content in the sample.

### A.6.4.2 Destruction using *microwave* or closed system destruction

a) Weigh 1 g of sample (m) into the tube destruction and add 5 ml $HNO_{3,}$ 1 ml $H_2O_2$ and then close the meeting.
b) enter into a *microwave* oven and work in accordance with the directions for use devices,
c) after cool, move the destruction solution to the volumetric flask 25 m quantitatively and dilute with distilled water until the line mark (V),
d) pipette 10 ml destruction solution into the borosilicate flask based round 50 ml, add 1 ml solution of Mg $(NO_{3)\ 2,}$ steaming above electric bath until dry and charchoal it. Ash in furnaces with a temperature of 450 ° C (± 1 hour),
e) chill, dissolved with 2.0 ml of HCl 8 M, 0.1 ml KI 20% and allow at least 2 minutes. Pour the solution into a sample tube on the instrument,
f) prepare $NaBH_4$ and HCl in a place in accordance with that determined by the instrument,
g) pour working standard solution As 0.01 µg / ml; 0.02 µg / ml; 0.03 µg / ml; 0.04 µg / ml; 0.05 µg / ml and the blank into the other 6 sample tubes. Light the burner and the reagents flow control knob and sample flow,
h) read the value of the highest absorbans of working standard solution As and sample with blank as correction,
i) create a calibration curve between the concentration of As (µg / ml) as X axis and absorbans as Y axis,
j) plot results of sample solution reading to the calibration curve (C),
k) do the duplo work, and
l) count content of As in the sample.

### A.6.5 Calculation

$$\text{Kandungan arsen (As) (mg/kg)} = \frac{C}{m} \times V \times fp$$

**Description:**

C is the concentration of arsenic from the calibration curve, expressed in micrograms per milliliter (µg / ml)
V is the volume of final solution, expressed in milliliters (ml);
m is the weight of sample, expressed in grams (g);
fp is the dilution factor.

### A.6.6 Accuracy

The range of twice test results is maximum 16% of the average content of arsenic (As). If the range is greater than 16%, then the analysis must be started again.

## A.7 Microbial contamination

### A.7.1 Preparation and homogenization of samples to test the total plate count, *escherichia coli*, mold, and yeast

#### A.7.1.1 Principle

Dismission of bacterial cells that may be shielded by the particles of food and to reactivate the bacterial cells that may be reduced its viability because of less favorable conditions in the food. Preparation and homogenization of samples aimed for good distribution of bacteria in food samples determined.

### A.7.1.2 Tools

a) appropriate Homogenization of the tools (blender) with a rotation speed of 10,000 rpm up to 12 000 rpm;
b) electric bath;
c) 2000 g capacity balance calibrated, with accuracy of 0.1 g;
d) calibrated volumetric flask 50 ml, 100 ml, 500 ml, dan 1 000.
e) sterile goblets;
f) sterile erlenmeyer flask;
g) sterile diluent bottles
h) Sterile volumetric pipette;
i) Test tube
j) knife, spoon, scissors, and sterile spatula.

### A.7.1.3 Diluent solution

*Butterfield's Phosphate-Buffered Dilution Water* (BPB);

- $KH_2PO_4$ 34 g
- distilled water 500 ml

Adjust pH with NaOH to pH 7.2, make exactly until 1000 ml volume with distilled water. Sterilization at a temperature of 121 ° C for 15 minutes. Store in refrigerator to make a diluent solution of 1.25 ml, stock solution diluted with distilled water until the volume of 1000 ml, then placed in diluent bottles of 450 ml and into a test tube as mauch as (9 ± 1) ml and sterilized at a temperature of 121 ° C for 15 minutes

### A.7.1.4 Sample homogenization

a) Weigh 50 g sample aseptically into erlenmeyer, which already contains 450 ml of diluent to obtain 1:10 dilution; and
b) Shake the mixture a few times so homogeneous.

## A.7.2 Total plate count (*plate count methods)*

### A.7.2.1 Principle

Mesophile aerobic bacterial growth after the sample was incubated in an appropriate seeding for 48 hours at a temperature (35 ± 1) ° C

### A.7.2.2 Tools

a) calibrated incubator cabinets;
b) oven / dry sterilizer calibrated;
c) autoclave;
d) *colony counter*
e) water bath;
f) measuring pipetter 1 ml, 5 ml, dan 10 ml steril; and
g) sterile glass/plastics petri dish diameter 15 mm x 90 mm

### A.7.2.3 Seeding and diluent

*Plate count agar* (PCA)

- *tryptone* 5 g
- *yeast extract* 2,5 g
- glucose 1 g
- agar 15 g
- distilled water 1 000 ml

Dissolve the above ingredients to 1000 ml with distilled water and adjust pH to 7.0. Enter into bottle. Sterilize using autoclave at a temperature of 121 ° C for 15 minutes.

### A.7.2.4 Workings

a) Create a level of dilution as needed as shown in Figure A.1 with the use of *Butterfield's phosphate-Buffered Dilution Water*

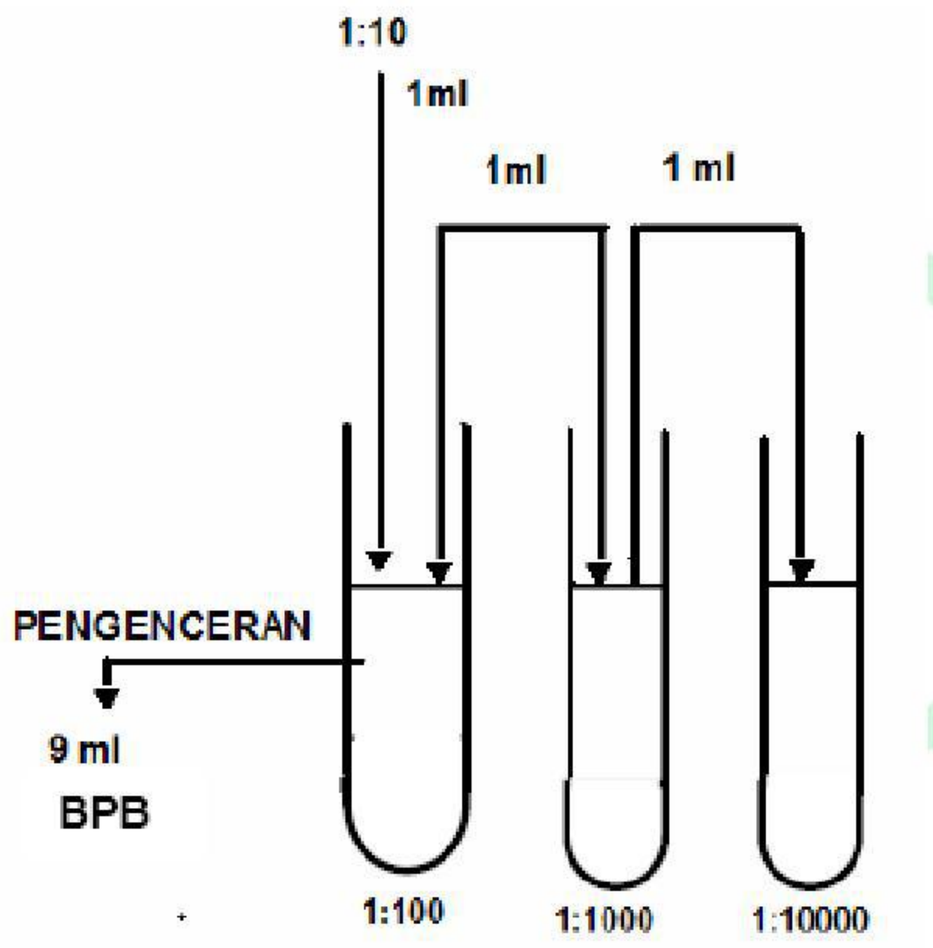


**Figure A.1 - The level of dilution**

b) pipette each 1 ml of 10 -1 dilution rate up to 10 -4 in sterile petri dish by duplo,
c) pour 12 ml to 15 ml PCA medium that is still liquid with a temperature (45 ± 1) ° C into each petri dish
d) shake petri dish carefully (swivel and rocking forward, backward, right and left) so that the samples and seeding mixed evenly and dense
e) blank checks done by mixing the diluent water for each sample tested, compact
f) let the mixture in a petri dish to solidify
g) enter all the petri dish upside down into incubator cabinet at a temperature of 35 ° C for (48 ± 2) hours, and
h) note the growth of colonies on each petri dish containing 25 colonies of up to 250 colonies after 48 hours.

### A.7.2.5 Calculation

$$\text{Angka lempeng total (koloni/g)} = n \times F$$

**Description :**

n is - average colony of two petri dish from one dilution, expressed as colonies per gram (colony / g);

F is the dilution factor of the average colony used

### A.7.2.6 Statement of result

### A.7.2.6.1 Calculating methods

a) select a petri dish from one dilution which shows the number of colonies between the 25 colonies to 250 colonies per petri dish. Count all the colonies in a petri dish using a colony counter. Calculate the average number of colonies and multiply by the dilution factor. Express the result as the number of bacteria per gram;

b) if one of two petri dish there is a smaller number of colonies from 25 colonies to or greater than 250 colonies, calculate the number of colonies located between the 25 colonies - 250 colonies and multiply by the dilution factor. Express the result as the number of bacteria per gram;
Example:

| $10^{-2}$ | $10^{-3}$ |
|---|---|
| 120 | 25 |
| 105 | 20 |

$$ALT = \frac{120 + 105 + 25}{[(1 \times 2) + (0{,}1 \times 1) \times 10^{-2}]} = 124{,}9375$$

c) if the result of two successive dilution number is located between the 25 colonies to 250 colonies, calculate the number of colonies from each dilution colonies per g by the formula:

$$ALT = \frac{\sum C}{[(1 \times n_1) + (0{,}1 \times n_2) \times d]}$$

**Description:**

C is the number of colonies from each dish;

$n_1$ is the number of dish from the first dilution which is calculated

$n_2$ is the number of dish from the second dilution;

d is the first dilution calculated;

Example:

| $10^{-2}$ | $10^{-3}$ |
|---|---|
| 131 | 30 |
| 143 | 25 |

$$ALT = \frac{131 + 143 + 30 + 25}{[(1 \times 2) + (0{,}1 \times 2) \times 10^{-2}]} = 164{,}3357$$

d) if the number of colonies from each dish more than 25 colonies declared as the approximate number of bacteria;
if the number of colonies per cm $^2$ of less than 100 colonies, then declare the result as approximate number: the number of bacteria multiplied by the dilution factor.
Example:

| $10^{-2}$ | $10^{-3}$ | Jumlah bakteri perkiraan |
|---|---|---|
| ~ | 640 | 1000 x 640 = 640.000 ($6{,}4 \times 10^5$) |

if the number of total colonies per cm $^2$ more than 100 colonies, then declare the result: area x dilution factor x 100 samples average number of colonies 110 per cm $^2$ example:

| $10^{-2}$ | $10^{-3}$ | area ($cm^2$) | jumlah bakteri perkiraan |
|---|---|---|---|
| ~ | 7150 | 65 | > 65 x $10^3$ x 100 = > 6500.000 (6,5 x $10^6$) |
| ~ | 6490 | 59 | > 59 x $10^3$ x 100 = > 5900.000 (5,9 x $10^6$) |

e) if the number of colonies of each colony that grew on the petri dish is less than 25, then express the approximate number of bacteria was less than 25 colonies multiplied by the lowest dilution; and

f) Calculating propagator colony;
Propagation in the colony have three kinds, namely:
- is a chain that does not separate;
- propagation which took place between the base and the seeding of the Petri dish; and
- propagation that occurs at the edge or surface of seeding.

If there is only one propagation (such as a chain) then the colony is considered one. If one or more chains are formed and come from separate sources, the steam source is calculated as one colony

### A.7.2.6.2 methods of calculating and rounding numbers

In reporting colonies number or tapproximate colonies number there are only two significant figures are used, namely the first and second digits (starting from left),

a) If the third digit is greater than 5, then round it off to the top;
for example: 528 reported as 530, and noted 5.3 x 10 $^2$
b) If the third digit is lessr than 5, then round it off to the bottom; and
for example: 528 reported as 520, and noted 5.2 x 10 $^2$
c) if the third digit equals 5, then round it off as follows
- for example: 528 reported as 560, and noted 5.8 x 10 $^2$
- Round it off to the bottom if the second number is an even number
for example: 565 reported as 560 , and note 905.6 x 10 2

## A.7.3 *Escherichia coli*

### A.7.3.1 Principle

Growth of *Escherichia coli* characterized by the formation of gas in Durham tubes, followed by biochemical tests and further referenced in Table A.2.

### A.7.3.2 Tools

a) incubator cabinet, (35 ± 1) ° C;
b) closed water bath with circulation systems, (45.5 ± 0.2) ° C;
c) racks for test tubes;
d) Mohr pipette 1 ml and 10 ml scale, and
e) dilution bottle (± 20 ml)-resistant Borosilicate glass, with a rubber stopper or screw cup
f) test tubes
g) Durham tube.
h) ose needle (inoculation), with a diameter approximately 3 mm;
i) i. glass petri dish size 15 mm x 100 mm or plastic size 15 mm x 90 mm, sterile;

### A.7.3.3 diluent and reagent seeding

a) *Lauryl sulfate tryptose* (LST) *broth* / *Lauryl tryptose* (LST) *broth* ;
b) *brilliant green lactose bile* (BGLB) *broth* 2 %;
c) EC *broth;*

d) *Levine's eosin methylene blue* (L-EMB) *agar* ;
e) *plate count agar* (PCA);
f) *gram stain* ;
g) *tryptone (tryptophane) broth* ;
h) *kovacs* reagent';
i) MR - VP *broth* ;
j) *voges proskauer* reagent ;
k) *methyl red* solution;
l) *koser's citrate broth* ;
m) *peptone diluents* 0,1 % *;*
n) *indol* e reagent;
o) 40% potassium hydroxide solution
p) *buffer fields phosfat buffered dilution water* ;
q) *alphanaphtol* , dan
r) *creatine monohydrate crystal* .

### A.7.3.4 Workings

#### A.7.3.4.1 APM – Prediction test for *Escherichia coli*

a. Perform preparation and homogenization of samples as in A.7.1,
b. inokulate each 1 ml solution of each level of dilution (solution of 10 -1, 10 -2 and 10 -3) into three tubes of LST broth. Hold the pipette so that the lower end of pipette attached to the tube. Allow the pipette contents to flow 2 seconds to 3 seconds Do not blow out the pipette contents,
c. insert tubes into the incubator at a temperature of 35 o C for (48 ± 2) hours,
d. observe these tubes on hour-(24 ± 2). If there is a tube that contains gas, state the tube is "positive",,
e. tubes that does not contain gas yet, stete "negative", continue the incubation for 24 hours,
f. note the existence of gas formation after incubation (48 ± 2) hours, and state the tube is "positive", and
g. do confirmation test against all the ‘positive’ tubes for prediction test.

#### A.7.3.4.2 APM – -- Confirmation test for *Escherichia coli*

a) Move one ose eye from each positive LST tube into a different tube of EC broth,
b) Inkubate the EC tubes into a circulating water bath, during (24 ± 2) hours on (45.5 ± 0.2) ° C, the tube that has formed gas is stated "positive", and
c) if negative, inkubaten and check back on hour-(48 ± 2). If the gas has been formed then the tube is stated "positive", and
d) do a complete test to all positive tubes for confirmation test b.

#### A.7.3.4.3 Complete test for *Escherichia coli*

a) Shake tubes positive EC carefully,
b) etched / implanted in an agar plate of L-emb, in such a way as to produce the separate colonies with a minimum distance of 0.5 cm
c) incubate L-EMB plate during the 18 hours up to 24 hours at (35 ± 1) ° C,
d) check fthe plates against the presence of green colonies with or without the flash of metal,
e) from each L-EMB plate, move up to five suspicious colonies on the tube in order to tilt the PCA,,
f) incubate tubes in order to tilt during the 18 hours up to 24 hours at 35 ° C and use for further testing,

g) make a Gram stain from each culture. E coli is a rod-shaped Gram-negative without spora that has to be tested using IMVIC reactions as below and should be inoculated back to the LST tubes to confirm the existence of gas production,

- the formation of indole
  - inoculation of tubes *tryptone broth* ,
  - incubation (24 ± 2) hours at 35 ° C,,
  - Test the existence of indole by adding 0.2 ml to 0.3 ml Kovacs', and
  - This test positive if the upper layer of red.
  - the formation of indole
- *Voges Proskauer* Test
  - Inoculation of medium tubes MR-VP from each PCA tube and incubate during (48 ± 2) hours at 35 ° C,
  - transfer apsetically 1 ml sterile test tube cultures,
  - add 0.6 ml of solution 5% in alcohol, 0.2 ml of KOH solution 40% and a few crystals of creatine, and
  - *Voges Proskaue*r test is positive if eosin pink color formed within 2 hours,
- *Methyl red* Test
  - After the VP test, incubate back MR-VP tube for 48 hours at 35 ° C,
  - add 5 drops of methyl red indicator on each tube, and
  - MR culture is considered positive if there is a red color, MR is negative when yellow.
- Citrate Utilization
  - Inoculate Koser's citrate broth tubes carefully using straight needles so that only on the surface of the medium. Too much inoculation can cause other substances carried
  - incubate for 96 hours at a temperature of 35 ° C, and
  - the growth in the tube as indicated by opaque color indicating a positive test.
- Gas forming from *Lactose*
  - Inoculate LST tubes from every oblique PCA. Inkubasikan during (48 ± 2) hours at a temperature of 35 ° C, and
  - check the tubes against the presence of gas formation.

### A.7.3.4.3.1 Classification and report

**Tabel A .1 - biochemical reaction *E.coli* in IMVIC test**

| Bodies | Indole | *Methyl Red* | *Voges Proskaeur* | Citrate |
|---|---|---|---|---|
| *Escherichia coli* | | | | |
| Vareities I | + | + | - | - |
| Vareities II | - | + | - | - |

- Classification as *E. coli* when IMVIC is + + - - or - + - -, gram coloring indicate rod shape negative gram without spora that forming gas in LST broth with incubation time (48 ± 2) hours at a temperature of 35 ° C

- Calculate the APM *E. coli* using Table A.2 APM based on the number of tubes from the three serial dilution that has been ascertained containing *E. coli*.

**Tabel A .2 - APM per 1 g sample if using 3 tubes**
**For each level of dilution 0,1 g/ml ; 0,01 g/ml ; and 0,001 g/ml sample**

| Positive tubes | | | APM | Positive tubes | | | APM |
|---|---|---|---|---|---|---|---|
| 0,1 | 0,01 | 0,001 | | 0,1 | 0,01 | 0,001 | |
| 0 | 0 | 0 | < 3 | 2 | 2 | 0 | 21 |
| 0 | 0 | 1 | 3 | 2 | 2 | 1 | 28 |
| 0 | 1 | 0 | 3 | 2 | 2 | 2 | 35 |
| 0 | 1 | 1 | 6 | 2 | 3 | 0 | 29 |
| 0 | 2 | 0 | 6 | 2 | 3 | 1 | 36 |
| 0 | 3 | 0 | 9 | 3 | 0 | 0 | 23 |
| 1 | 0 | 0 | 4 | 3 | 0 | 1 | 39 |
| 1 | 0 | 1 | 7 | 3 | 0 | 2 | 64 |
| 1 | 0 | 2 | 11 | 3 | 1 | 0 | 43 |
| 1 | 1 | 0 | 7 | 3 | 1 | 1 | 75 |
| 1 | 1 | 1 | 11 | 3 | 1 | 2 | 120 |
| 1 | 2 | 0 | 11 | 3 | 1 | 3 | 160 |
| 1 | 2 | 1 | 15 | 3 | 2 | 0 | 93 |
| 1 | 3 | 0 | 16 | 3 | 2 | 1 | 150 |
| 2 | 0 | 0 | 10 | 3 | 2 | 2 | 216 |
| 2 | 0 | 1 | 14 | 3 | 2 | 3 | 290 |
| 2 | 0 | 2 | 20 | 3 | 3 | 0 | 240 |
| 2 | 1 | 0 | 15 | 3 | 3 | 1 | 460 |
| 2 | 1 | 1 | 20 | 3 | 3 | 2 | 1100 |
| 2 | 1 | 2 | 27 | 3 | 3 | 3 | > 1100 |

### A.7.4 *Salmonella sp.*

#### A.7.4.1 Principle

Samples which were tested were grown first on the enrichment medium and then grown on selective media. Further, samples detected by grow on selective agar media. Colonies that suspected Salmonella sp. on selective media then isolated and confirmated by biochemical test and serological tests to assure the presence or absence of Salmonella sp.

#### A.7.4.2 Tools

a) incubator, (35 ± 2) ° C;
b) Refrigerated incubator or laboratory refrigerator, (4 ± 2) ° C
c) autoclave;
d) oven, oven;

e) water bath, (49 ± 1) ° C;
f) water bath, circulating, thermostatically-controlled, (43 ± 0.2) o C;
g) water bath, circulating, thermostatically-controlled, (42 ± 0.2) o C;
h) balance, capacity 2000 grams, with precision 0.1 grams;
i) balance, capacity 120 grams, with precicion 5 mg;
j) blender with a rotation speed 10000-12000 rpm and a blender jar (bottle) sterile;
k) wide-mouthed bottle screw cap 16 oz (500 ml) sterile, Erlenmeyer 500 ml sterile, beaker 250 ml sterile, sterile glass or paper funnels to the size appropriate, and, more options, container with appropriate capacity to accommodate composite sample;
l) Bent glass or plastic spreader bars sterile;
m) sterile spoon, or other equipment to move the food samples;
n) sterile petri dish, 15 x 100 mm, glass or plastic;
o) sterile pipette, 1ml with accuracy 0.01 ml, and sterile pipette 5 and 10 ml with 0.1 ml accuracy;
p) ose needle (inoculation) (± 3 mm diameter), nichrome, platinum-iridium Chromel wire or sterile plastic;
q) test tubes or sterile culture tubes, 10 x 150 mm and 20 x 150 mm; serological tubes, 10 x 75 mm or 13 x 100 mm;
r) test tube racks or culture tube racks;
s) vortex mixer;
t) sterile scissors, large scissors, scalpels, and forceps ;
u) light (to observe the serological reactions);
v) fisher or a Bunsen burner;
w) pH paper (range pH 6-8) with a maximum accuracy of 0.4 pH units per color change;
x) pH meter;
y) sterile plastic bag, 28-37 cm and can be tied;
z) plastic beaker, 4 liters, can be autoclaved, to prop up the plastic bag during shaking and incubation;
aa) sponges, non-bactericidal (Nasco paint # B01299WA) or comparable; and
bb) swab, non-bactericidal, types of cotton.

### A.7.4.3 Seed and reagents

a) *Lactose broth* ;
b) *tetrathionate* (TT) *broth* ;
c) *Rappaport-Vassiliadis* (RV) *medium* (RV *medium* must be made from materials contained in the composition of the RV medium. Commercially available formulations can not be accepted);
d) *xylose lysine desoxycholate* (XLD) *agar* ;
e) *hektoen enteric* (HE) *agar* ;
f) *bismuth sulfite* (BS) *agar* ;
g) *triple sugar iron* (TSI) *agar* ;
h) *tryptone* ( *tryptophane* ) *broth* ;
i) *trypticase* ( *tryptic* ) *soy broth* ;
j) *trypticase soy broth* dengan *ferrous sulfate* ;
k) *trypticase soy-tryptose broth* ;
l) *methyl red* -Voges Proskeaur (MR-VP) *broth*
m) *simmons citrate agar* ;
n) *urea broth* ;
o) *urea broth* ( *rapid* );
p) *malonate broth* ;
q) *lysine iron agar* (LIA) (Edward dan Fife)
r) *lysine decarboxylase broth* ;
s) motility test medium (semi solid) ;

t) *potassium cyanide* (KCN) *broth* ;
u) *phenol red carbohydrate broth* ;
v) *purple carbohydrate broth* ;
w) *MacConkey agar* ;
x) *nutrient broth* ;
y) *brain heart infusion* (BHI) *broth* ;
z) papain solution , 5 %;
aa) Cellulose solution, 1 %;
bb) *tryptose blood agar base* ;
cc) powdered *potassium sulfite, anhidrous* ;
dd) chlorine solution, 200 ppm, contains 0,1 % *sodium dodecyl sulfate* ;
ee) ethanol 70 %;
ff) Kovacs reagent;
gg) Voges-Proskauer (VP) test reagent;
hh) Crystal *creatine phosphate* ;
ii) *potassium hydroxide* solution, 40 %;
jj) *sodium hydroxide* solution 1 N;
kk) hydrochloric acid 1 N;
ll) *brilliant green dye* solution, 1 %;
mm) larutan *bromcresol purple dye* , 0,2 %;
nn) indicator *methyl red* ;
oo) sterile distilled water;
pp) *tergitol anionic* ;
qq) *triton* X-100;
rr) *physiological saline* solution, 0,85 % (sterile);
ss) *formalinized physiological saline* solution ;
tt) *Salmonella polyvalent somatic* (O) *antiserum* ;
rr) *Salmonella polyvalent flagellar* (H) *antiserum* ;
vv) *Salmonella somatic group* (O) *antisera* : A, B, $C_1$ , $C_2$ , $C_3$ , $D_1$ , $D_2$ , $E_1$ , $E_2$ , $E_3$ , $E_4$ , F, G, H, I, Vi, or other appropriate group;
ww) *Salmonella* Spicer-Edwards *flagellar* (H) *antisera* ;

### A.7.4.4 Workings

#### A.7.4.4.1 Sample homogenization and pre-enrichment

a) Weigh 25 g sample aseptically into sterile 500 ml bottle and add 225 ml sterile lactose broth;
b) shake gently and leave at room temperature for (60 ± 5) minutes with a bottle in a closed state;
c) shake by twirling the bottle carefully and determine the pH using pH paper
d) if necessary, adjust the pH by adding 2.25 ml of anionic tergitol 7 becomes (6.8 ± 0.2);
e) adjustment of pH may also be made by adding 2 to 3 drops of triton X-100;
f) loosen the container lid adequately (about ¼ turn) and incubate during (24 ± 2) hours at 35 ° C

#### A.7.4.4.2 Enrichment

a) Tighten the lid and shake gently the sample that have been incubated

b) pipettes 0.1 ml pre-enrichment culture into 10 ml Rappaport-Vassiliadis (RV) medium and 1 ml other pre-enrichment culture into 10 ml of tetrathionate (TT) broth and vortex the mixture respectively, and

c) incubate Rappaport-Vassiliadis (RV) medium at a temperature (42 ± 0.2) ° C (24 ± 2) hours and tetrathionate (TT) broth at (35 ± 2.0) ° C for (24 ± 2) hours in circulating water bath.

### A.7.4.4.3 Planting on selective seedling

a) Shake the samples that have been incubated and by using ose needle, scratch along the 3-mm TT enrichment culture into petri dish containing medium of XLD, HE and BS agar. Prepare BS agar for a day before use and store in a dark place at room temperature until ready to be scrathed

b) repeat the above method from RV enrichment medium

c) incubate dish containing BSA, HE and XLD media during (24 ± 2) hours at a temperature of 35 ° C, and

d) observe the presence possibility of Salmonella sp colony.
Colony morphology has the following characteristics:
Take two or more colonies of Salmonella sp. from each selective agar media after (24 ± 2) hours of incubation. Colonies of Salmonella sp. are as follows:

XLD : pink colonies with or without a black core. Most Salmonella sp. colony forming large, shiny black core or it may appear almost entirely black. Some cultures of Salmonella sp. produce yellow colonies with or without a black core in the media XLD and HE.

HE : bluish-green colony until the blue with or without a black core. Most Salmonella sp. forming large colony, shiny black core or it may appear almost entirely black.

BS : brown colony, gray to black and the occasionally metallic shiny. If the incubation period increases the color of the media around the colonies at first brown then black. In some strains of green colonies with or without a dark color around the media.

e) if no typical colonies or suspect colonies on BSA medium after incubation (24 ± 2) hours, do not take the colonies but the incubate back the medium during (24 ± 2) hours. If no typical colonies or suspect colonies on BSA medium after incubation (48 ± 2) hours, take two or more colonies that are not typical,

f) using a sterile inoculation needle, carefully take the middle colonies and inoculate into TSI medium ablique agar by scratch ablique agar, and stabbed to erect. Without taking a new colony, use the same needle to inoculate LIA by piercing so straight ahead, after it scratched on that ablique agar, de. Because Lysine decarboxylase reaction is anaerobic, LIA italics must have a deep puncture (4 cm). Save the selective agar media that has taken the colony at a temperature (5 ± 8) ° C,

g) Incubate TSI and LIA at temperature 35 ° C for (24 ± 2) hours by leaving the lid slightly loosened to prevent the formation of $H_2S$ that is redundant. At TSI, typical Salmonella sp. culture gives an alkaline reaction (red) on the scratches and acid (yellow) at the puncture, with or without $H_2S$ (blackish color on agar). At LIA typical Salmonella sp. culture gives an alkaline reaction (purple) in the whole tube. The reaction which is really yellow in the puncture is expressed as a negative culture. Do not just look at the changing colors of the puncture to state a negative culture. Generally Salmonella sp. culture forming $H_2S$ in in LIA obligque agar, Some non-Salmonella sp culture. form a brick red reaction in LIA obligque agar,

h) all the cultures that give an alkaline reaction on the part of a puncture in the media of LIA with regardless of TSI reaction will potentially as Salmonella sp. and conducted biochemical and serological tests. Cultures that give acid reaction to a puncture on LIA and alkaline media on goresannya and acid reaction to a puncture in the TSI should be considered as well as the potential of Salmonella sp. and must be carried out biochemical and serological. Cultures that provide acidic reaction on puncture in the

LIA medium and acid on its scrathes, and the acidic reaction on its puncture in TSI Medium can be stated as not Salmonella sp. If TSI cultures do not show typical reactions of Salmonella sp. (Alkaline in the scratches and acid on the puncture), repeat the test by taking a suspicious colonies from selective medium that does not give positive predictive culture and inoculation by scratching TSI and LIA media such as how to start section f above,

i) do biochemical and serological identification tests to:

- three TSI presumtif cultures from one set of selective media (HE, XLD and BSA) which is innoculated from Lp, and three presumtif cultures which is inoculated from RV,
- if the three positive presumtif culture of TSI not isolated from one set of selective media, test the TSI positive presumtif from other agar media. Test at least six TSI cultures for each 25 g sample of food.

### A.7.4.5 Identification of *Salmonella sp.*

#### A.7.4.5.1 Mixed culture

a) If the culture of TSI Agar be seen mixed , then scratched back into the MacConkey agar media, HE or XLD. Incubate for (24 ± 2) hours at a temperature of 35 ° C. Observe colonies suspected Salmonella sp.

- *Mac Conkey agar* . Typical colonies appear transparent and colorless, sometimes with a black core. Colonies of Salmonella sp. will form a bright area due to the deposition of other bacteria that sometimes grows
- *hektoen enteric* (HE) agar. Bluish green colonies until the blue-black with or without core.. In general culture of Salmonella sp. colony forming large, shiny black core or almost all colonies visible black
- *xylose lysine desoxycholate* (XLD) agar. Pink colonies (pink) with or without black core. In general culture of Salmonella sp. colony forming large, shiny black core or almost all colonies visible black.

b) Move at least two colonies suspected Salmonella sp. In TSI and LIA imedia as determined in A.7.4.4.3.f and continue in accordance with A.7.4.4.3.g

#### A.7.4.5.2 Pure culture

a) urease test (conventional)

Inoculate from TSI suspected *Salmonella sp.* with inoculation needles into *Urea broth* . Incubate during (24 ± 2) hours at a temperature of 35 °C, and

b) urease test (fast).

Inoculate from TSI suspected *Salmonella sp.* With inoculation needle into *rapid urea Broth* . Inoculate for 2 hours in water bath at a temperature of (37 ± 0,5) °C. Typical Reaction of *Salmonella sp.* For urease test gave negative results (no color change).

#### A.7.4.5.3 Test of negative urease culture

a) *lysine Decarboxylase Broth* (LDB)

This test is carried out only if the reaction of LIA is dubious .Take a ose from TSI and inoculate into LDB Media. Loosen the lid and incubate for (48 ± 2) hours at a temperature of 35 ° C, but observed after 24 hours . *Salmonella sp.* give alkaline reactions indicated by the color purple in all media. Negative reaction is shown in yellow colour in all media. If the results doesnot show the color yellow or purple, add a few drops of 0.2% *bromocresol purple dye* and observe the color changes, ,

b) *phenol red dulcitol* or *purple broth base* with 0,5 % *dulcitol* , and

Inoculatei *dulcitol broth* media andi TSI cultures, loosen the lids and incubate for (48 ± 2) hours at a temperature of 35 °C and observe after 24 hours. In general *Salmonella sp.* gives positive result indicated witg formation of gas in Durham tubes and acid pH (yellow) in the media. Negative reactions are not characterized by the formation of gas in Durham tubes and red (phenol red as indicator) or purple (bromocresol purple as indicator) on all media.

c) *tryptone (tryptophane) broth* (TB),

Inoculate *tryptone broth* media and biakan TSI cultures. Incubate for 24 hours at a temperature of 35 °C and then follow procedures belowi:

- *Potasium Cyanida* (KCN) *broth*
- Move 1 ose cultures arid TB 24 hours into the KCN *broth* media. Close the jar tightly and seal with parafilm paper. Incubate for (48 ± 2) hours at a temperature of 35 °C bot observe after 24 hours. Positive results shown by the presence of growth (indicated by the turbidity). In general *Salmonella sp.* do not grow on this media characterized by not occurring turbidity
- *malonate broth*

  Move a ose of TB culture into *Malonate Broth*. Media. Incubate for (48 ± 2) hours at a temperature of 35 °C, but observe after 24 hopurs. Sometimes *Malonate Broth* tubes that not to be inoculated turn blue. Therefore, use *Malonate broth* as a control. positive reaction is indicated by color changes to blue. Generally, *Salmonella sp.* gives negative reaction (no green or discoloration) on this *broth*. .
- Indole test

  Of the remaining TB medium, add 0.2 ml to 0.3 ml with *Kovacs*' reagents. Observed immediately after addition of reagents. Positive reaction indicated by the formation of a red ring on the surface of the media. Generally, *Salmonella sp*. gives negative reaction (no red ring formed on the surface of the media). The reaction that the color is between orange and pink expressed as positive.

State cultures as not *Salmonella sp.* if the Indol reaction is positive and *flagellar* (H) is negative, or KCN is positive and LDB is negative.

### A.7.4.5.4 Serological test of *polyvalent flagellar* (H)

a) Inoculate from each TSI agar which provide a negative urease reaction into:
   - *BHI broth* BHI broth, and incubate for 4 hours up to 6 hours at a temperature of 35 ° C until visible growth (to be tested on the same day) or
   - *Trypticase Soy Trypt o se Broth* (TSTB),) and incubate for (24 ± 2) hours at a temperature of 35 ° C (to be tested the next day). Add 2.5 ml of formanilized physiological saline solution into 5 ml of culture above,

b) Prepare 2 cultures from TSI (sample and control) that have added with *formanilized physiological saline* and test with *Salmonella sp. polyvalent flagellar* (H) antisera. Enter a ± 0.5 ml saline Salmonella sp. polyvalent flagellar (H) antisera solution into serology tubes 10 mm x 75 mm or 13 mm x 100 mm. Add 0.5 ml of antigen to be tested. Prepare the saline control by mixing 0.5 ml of formanilized physiological saline with 0.5 formanilized ml antigen. 0,5 ml *formanilized* antigen. Incubate the mixture in a water bath at a temperature of 48 ° C to 50 ° C. observe every interval time of 15 minutes and observe the results in one hour.
   - Positive if there is clumping in the test mixture and no clumping in the control,
   - Negative if no clumping in the test mixture and in control, and
   - Non-specific there is cliumping in the test mixture and in control

### A.7.4.5.5 Serological test of *polyvalent somatic* (o)

a) Use a wax pencil, create a rectangle 1 cm x 2 cm above the glass or plastic petri dish 15 mm x 100 mm or above the glass preparation,
b) emulsify cultures from TSI Oblique age of 24 hour t up to 48 hours with 2 ml of 0.85% saline using ose needle (can also use the culture of t*ryptose blood agar base* without blood),
c) add a drop of cultures suspension over each part of rectangle that has been marked with a wax pencil,
d) add a drop of saline solution in the first section and add a drop of *polyvalent somatic (O)* antisera into another part,
e) mix or homogenize top section using clean and sterile ose during 1 minute, and
f) Classification of *polyvalent somatic* (o) test show the results as follow:
   – Positive : there is clumping in test mixture, no clumping in *saline* control
   – Negative :no clumping in test mixture and *saline* control,
   – Non-specific there is clumping in test mixture and *saline.* control

### A.7.4.5.6 Additional biochemical tests

State as *Salmonella sp.* , cultures that give typical reaction as on the Table A.3 number 1 upto 11. If 1 TSI cultures from dari each of 25 g sample indicate *Salmonella sp.* , additional biochemical tests are not necessary Culture that gave a positive reaction in serologic tests of flagellar (H) but do not show characteristics of Salmonella sp. on biochemical tests, must be purified according to A.7.4.5.1 above and test again as specified in A.7.4.5.2

Perform the following additional test against a culture that does not give the typical reactions such as Table A.3 :

a) *Phenol red lactose* or *purple lactose broth*
   Inoculate this broth wit oblique TSI agar cultures that had incubated for 24 upto 48 hours. Incubate for (48 ± 2) hours at a temperature of 35 °C, but observe after 24 hours,
   – Positive, in case of formation of acid (yellow) and gases in Durham tubes. hen only the formation of acid, it can be stated positive. In general *Salmonella sp.* gives negative results, indicated by no gas formation in the Durham tube and red colour ( *phenol red* as indicator) or purple *bromocresol purple* as indicator in all media
   – If cultures give lactose positive reaction, state as not *Salmonella sp.* , except for cultures gave acid reaction in oblique agar TSI and alkaline reaction in LIA or positive reaction in *malonate broth* ,
b) *Phenol red sucrose* or *purple sucrose broth*
   Follow procedures in accordance with A.7.4.5.6.a. Declare it as not *Salmonella sp*. in the culture gave a positive reaction, except for cultures that give acid reactions on oblique agar TSI and a positive reaction (alkaline) in the LIA,
c) *Methyl Red-Voges-Proskauer* (MR-VP) *broth*
   Inoculate the medium with e few oblique agar TSI cultures. Incubate for (48 ± 2) hours at a temperature of 35 °C,
   Do *Voges-Proskauer* (VP) test at room temperature as folllow:
   Move 1 ml MR-VP Broth that has been incubated for (48 ± 2) hours into sterile test tubes and incubate again the MR-VP Broth during (48 ± 2) hours at a temperature of 35 ° C
   – Add 0,6 ml alpha naphtol and stir,

- Add 0.2 ml of KOH solution 40% and stir again. Fpr accelerating the reaction add a few creatine crystals and observe the results after four hours, and
- Change of color to be red brick until red ruby red on the media indicate a positive reaction. Generally, *Salmonella sp.* give negative VP reaction

Methyl red test (MR)

- Add 5 drops of methyl red indicator into the MR-VP media that have been incubated for 96 hours,
- observe the results immediately, and
- Generally *Salmonella sp.* gave a positive reaction, marked by the diffusion of the red color in the media. Yellow colors indicate the occurrence of negative reaction,

State as not *Salmonella sp.* the culture that gave KCN, and VP positive reaction, and MR negative reaction

d) *Simmons citrate agar*

- Inoculate media using a ose-needle containing cultures of the oblique TSI agar , by scraping oblique agar. Inkubate during (96 ± 2) hours at a temperature of 35
- positive, if there is growth that usually followed by a color change from green to blue. Generally, Salmonella sp. gave positive citrate test results
- negative if no or very little growth and no color change

### A.7.4.5.7 Statement of Results

State as *Salmonella sp.* cultiures that have such reactions in Tabel A.3. When no one of TSI culture that shows reaction of *Salmonella sp.* on biochemical test, do biochemical test startt from A.7.4.5.3 on culture that gives a negative urease reaction from the same sample.

**Table A .3 - Biochemical and serology reaction for Salmonella sp.**

| No | Test or substrate | Resultant | | *Salmonella sp.* Reaction species [a] |
|---|---|---|---|---|
| | | Positive | Negative | |
| 1 | *glucose* (TSI) | yellow prick | red prick | + |
| 2 | *lysine decarboxylase* (LIA) | purple prick | yellow prick | + |
| 3 | $H_2S$ (TSI dan LIA) | black | Not black | + |
| 4 | urease | purple to red | no color change | - |
| 5 | *lysine decarboxy broth* | purple | yellow | + |
| 6 | *phenol red dulcitol broth* | yellow and/or gas | No gas formation, no color change | +[b] |
| 7 | KCN *broth* | growth | No growth | - |
| 8 | *malonate broth* | purple | no color change | -[c] |

| Table A.3 (continued) | | | | |
|---|---|---|---|---|
| **No** | **Test or substrate** | **Resultant** | | ***Salmonella sp.*** **Reaction species** [a] |
| | | **Positive** | **Negative** | |
| **9** | *indole test* | surface color indigo | yellow surface | **-** |
| **10** | *polyvalent flagellar test* | clumping | no clumping | **+** |
| **11** | *polyvalent somatic test* | clumping | no clumping | **+** |
| **12** | *phenol red lactose broth* | yellow and/or gas | No gas is formed and no color change | **-**[c] |
| **13** | *phenol red sucrose broth* | yellow and/or gas | No gas is formed and no color change | **-** |
| **14** | *voges-proskauer test* | Pink to red | no color change | **-** |
| **15** | *methyl red test* | Spread red | Spread yellow | **+** |
| **16** | *simmons citrate* | Growth, blue | No growth and no color change | **V** |

**Description:**
[a] : + 90% or more positive in opne or two days;
- : 90% or more negative in one or two days;
V : variable
[b] : majority of Salmonella arizonae cultures: negative
[c] : majority of Salmonella arizonae cultures: positive

**Tabel A .4 - Biochemical and serology reaction for non Salmonella sp**

| No | Test or substrate | Results |
|---|---|---|
| **1** | *urease* | Positive (purple-red) |
| **2** | *Indole test* and *polyvalent flagellar (H) test* | positive (surface color indigo)<br>negative(no clumping) |
| | or *Indole test* and *Spicer-Edwards flagellar test* | positive (surface color indigo)<br>negative (no clumping) |
| **3** | *lysine decarboxylase* and KCN *broth* | negative (yellow)<br>positive(growth) |
| **4** | *phenol red lactose broth* | positive (yellow and/or gas) [a,b] |
| **5** | *phenol red sucrose broth* | positive (yellow and/or gas) [b] |
| **6** | KCN *broth* , *Voges-pro ska uer test* and *methyl red test* | positive (growth)<br>positive (pink to red)<br>negative (yellow spread) |

**Description:**
[a] : *malonate broth* test is positive when the culture is *Salmonella arizonae*
[b] : do not waste positive cultur if LIA culture indicate a reaction characterized *Salmonella sp.* , Further tests to examine whether the species is Salmonella sp.

### A.7.5 Molds and yeasts

#### A.7.5.1 Principle

Growth of mold and yeast within relevant mediai, after incubation at a temperature of u (25 ± 1) °C during 5 daysi.

#### A.7.5.2 Tools

a) Calibrated incubator 25 °C;
b) autoclave;
c) water bath (45 ± 1) °C;
d) colony counter;
e) microscope;
f) petri dish 15 mm x 100 mm; and
g) Measuring pipette 1 ml and 10 ml.

#### A.7.5.3 Breeding and diluent

Choice of media::

a) Media with the addition of antibiotic solution ,
   - *dichloran rose bengal chloramphenicol* (DRBC) agar,
   - *dichloran* 18 % *glycerol* (DG 18) agar,

   antibiotics added to the media mold and yeast to prevent bacterial growth... *Chloramphenicol* is one antibiotic option, because it is stable when being autoclaved. Antibiotic concentration allowed is 100 mg per liter of media. If visible growth of bacteria, prepare the media by the addition of 50 mg per liter *chloramphenicol* before the autoclave and 50 mg per liter of sterilized *chlortetracycline* when the media began to be conditioned, just before pouring the media in the cup.
b) *plate count agar* (PCA);
   add 100 mg chloramphenico*l* per liter, if using this media. This media is not suitable if there is suspected molds spread (sample M ucor, R hizopus etc.);
c) *malt agar* (MA);
d) *malt extract agar (* molds and yeast) (MEAYM); or
e) *potato dextrose agar* (PDA):
   - infusion and white potatoes 200 g
   - *dextrose* 20 g
   - agar 20 g
   - distilled water 1 000 ml

Dissolve all the ingredients above. Enter the pumpkin, sterilize at 121 ° C for 15 minutes Before use cool until 50 ° C and pH 3.5 is set with 10% sterile tartaric acid. Decrease in pH can be changed with the addition of 4 ml of antibiotics (1g/100ml). Mix, then pour into a petri dish.

#### A.7.5.4 workings

a) Perform sample preparation and homogenization in accordance with A.7.1,
b) There are two methods of media preparation in the cup, :
   - method of spread in the cup (for media selection DRBC and DG 18):
     pipette 0.1 ml of each dilution aseptically into the solid medium and spread evenly using a glass rod.
   - method of spread in the cup (for media selection DRBC and DG 18):

pipette 1.0 ml of each dilution into a petri dish 15 mm x 100 mm and immediately pour 20 ml to 25 ml of media. Mix by gently shaking the cup clockwise, then counterclockwise within 1 to 2 minutes.

c) allow it to freeze the mixture in a petri dish,
d) pipette each 1 ml of dilutions $10^{-1}$ to $10^{-2}$ into a sterile Petri dish in duplo,
e) Enter all the Petri dish with no upside down position into the incubator and incubation in a dark room with a temperature of 25 ° C for 5 days,
f) count colonies of mold and yeast (the calculation can be done from day three to day five). If after five days no growth, add incubation time for 48-hour, and
g) state the results of calculation as the amount of mold and yeast per gram of sample.

### A.7.5.5 Statement of results

#### A.7.5.5.1 Methods of calculating

How to calculate the mold / yeast, such as how to calculate the total plate count

#### A.7.5.5.2 Methods of calculating and rounding numbers

How to calculate and round numbers such as how to mold and yeast count and round off numbers on total plate count.

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